PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants: Goelet *et al.*

Docket No.: 13020-2(DIV1)

Serial No.: Not yet assigned

Examiner (from parent): Sisson, B.

Filed: Herewith

Group Art Unit (from parent): 1655

For: SINGLE NUCLEOTIDE POLYMORPHISMS AND THEIR USE IN GENETIC ANALYSIS

Kalow & Springut LLP
488 Madison Avenue, 19th Floor
New York, NY 10022

May 1, 2001

Assistant Commissioner for Patents
Washington, DC 20231

AMENDMENT

Sir:

Prior to examination on the merits, please amend the application identified above as follows:

IN THE SPECIFICATION

At page 1, line 18, immediately after the phrase "This application is" please insert: --a divisional application of U.S. Patent Application Serial No. 08/971,344 (filed November 17, 1997), which is a continuation application of U.S. Patent Application Serial No. 08/216,538 (filed March 23, 1994), which is--

At page 1, line 19, immediately after the phrase "(filed November 3, 1993)" please insert: --and abandoned January 11, 1996). All of these applications are herein incorporated by reference.--.

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5/1/01
Date

J. Colwell
Name

At page 9, line 4, please replace the word "us" with the word --is--.

At page 10, line 30, please replace the word "trialleleic" with the word --triallelic--.

At page 11, line 31, please replace the word "Correspoinding" with the word - -Corresponding--.

At page 12, line 21, please delete the word "the".

At page 12, line 25, please replace the word "An" with the word - -A--.

At page 15, line 17, please replace the phrase "WO 92/15712, herein incorporated by reference" with the phrase --WO 92/15712--.

At page 22, line 3, please replace the word "is" with the word --are--.

At page 25, lines 3-4, please delete the phrase "(U.S. patent application serial no. 08/005,061, herein incorporated by reference).

At page 25, after line 6, please insert the following:

-- The invention as described in application serial No. 08/005,061 provides a method for generating single-stranded DNA molecules following a primer-mediated extension or amplification reaction. Such molecules are useful as hybridization probes and in nucleic acid sequencing.

In detail, the invention as described in application serial No. 08/005,061 provides a

method for generating a desired single-stranded nucleic acid molecule having a sequence complementary to that of a target nucleic acid molecule, the method comprising the steps:

a) incubating the target molecule in the presence of a primer molecule; wherein the primer molecule is capable of hybridizing to the target molecule, and wherein the primer molecule contains a nucleotide that is substantially incapable of being eliminated by a 5' → 3' exonuclease;

b) permitting template-dependent extension of the primer molecule to thereby form the desired nucleic acid molecule; and

c) incubating the target molecule in the presence of a 5' → 3' exonuclease, wherein the incubation results in the elimination of the target molecule, and thereby generates the desired single-stranded molecule.

The invention as described in application serial No. 08/005,061 additionally concerns the embodiment of the above method wherein step B additionally includes the substep of incubating the desired nucleic acid molecule in the presence of a second primer molecule capable of hybridizing thereto, and of being extended in a template-dependent manner to thereby form a nucleic acid molecule having a sequence substantially complementary to that of the desired molecule.

The invention as described in application serial No. 08/005,061 additionally concerns the embodiment of the above method wherein step B additionally includes the further substeps of hybridizing the nucleic acid molecule of step B, substep (1), with a complementary primer molecule; and permitting template-dependent extension of the complementary primer molecule to form a nucleic acid molecule having a sequence substantially complementary to that of the target molecule.

The invention as described in application serial No. 08/005,061 additionally concerns the embodiment of the above methods wherein the primer, the phosphorothioate nucleotide derivative, or the single-stranded molecule, or its amplification product is detectably labeled, as with an enzyme label, a fluorescent label, a radioisotopic label, and a chemiluminescent label.

The invention as described in application serial No. 08/005,061 additionally concerns the embodiment of the above methods wherein the desired single-stranded nucleic acid molecule or amplification products are detectably labeled by the incorporation of labeled nucleotides during the template-dependent extension of the primer.

The invention as described in application serial No. 08/005,061 is capable of generating single-stranded molecules regardless of the nature, origin or sequence of the target molecule. Thus, the invention as described in application serial No. 08/005,061 can be used to generate single-stranded molecules that have a naturally occurring sequences, such as a sequence present in a virus (e.g. rhinovirus, hepatitis virus, herpes virus, HIV, etc.), a bacterium (e.g. Escherichia, Clostridium, Mycobacterium, Neisseria, Mycoplasma, Vibrio, Chlamydia, Rickettsia, etc.), a yeast, a fungus, or other lower eukaryote. In particular, the present invention can be used to generate single-stranded molecules that have sequence present in a plant cell, or an animal cell (especially a mammalian cell, such as from a horse, cow, dog, cat or human). The invention as described in application serial No. 08/005,061 can also be used to generate single-stranded molecules that are purely or partially synthetic (i.e. non-naturally occurring). --

At page 25, line 7, please delete "incorporated by reference".

At page 31, line 3, please replace the term "Tween-20" with the term --polyoxyethylenesorbitan-20 (TWEEN-20)--.

At page 31, line 13, please replace the term "Tween-20" with the term -- (TWEEN-20)--.

At page 33, lines 27-28, please replace the phrase "WO 92/15712, herein incorporated by reference" with the phrase --WO 92/15712--.

At page 50, line 17, please replace the word" is" with the word --are--.

At page 51, line 14-17, please delete the phrase "First, the amplification may be mediated using primers that contain 4 posphorothioate-nucleotide derivatives, as taught by Nikiforov, T. (U.S. patent application serial no. 08/005,061)." and replace it with -- First, the amplification may be mediated using primers that contain 4 phosphorothioate-nucleotide derivatives.--

At page 52, line 11, please replace the phrase "20 l" with the phrase --20 µl--.

At page 52, line 13, please replace the phrase "Twenty l" with the phrase --Twenty µl--.

At page 52, line 15, please replace the phrase "per l" with the phrase --per µl--.

At page 52, line 15, please replace the phrase "polymerase and incubated for" with the phrase -- polymerase) was added to each well and incubated for--.

At pages 61-62, please delete the section entitled "False Report Rate" which begins on page 61, line 3 and ends on page 62, line 21.

At page 62, after line 21, please insert the following:

--**EXAMPLE 7**

GENETIC BIT ANALYSIS

DNA Samples. Genomic DNA was isiolated using the SDS/Proteinase K procedure (Maniatis, T. Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, 1989) from peripheral blood nucleated cells of humans or horses enriched from red blood cells by selective lysis accomplished by diluting blood with a three fold volume excess of ACK lysing buffer (0.15 M ammonium chloride, 1 mM potassium bicarbonate, 0.1 mM EDTA). Oligonucleotides were prepared by solid-phase phosphoramidite chemistry using an Applied Biosystems, Inc. (Foster City, CA) Model 391 automated DNA synthesizer. In the case of primers used in Genetic Bit Analysis (GBA) reactions, detritylation was not performed following the final cycle of synthesis and the full-length oligonucleotide was purified using the Applied Biosystems oligonucleotide purification cartridge (OPC) as recommended by the manufacturer. For most PCR reactions, primers were used directly by drying down the de-protection reaction. Oligonucleotides derivatized with 5'-amino groups were prepared using Aminolink 2 purchased from Applied Biosystems and used according to the manufacturer's recommendations.

Template Preparation. Amplification of genomic sequences was performed using the polymerase chain reaction (PCR) (Saiki, R.K., Gelfand, D.H., Stoffel, S., Scharf, S.J., Higuchi, R., Horn, G.T., Mullis, K.B., Erlich, H.A., Primer Directed Enzymatic Amplification of DNA with a Thermostable DNA Polymerase. Science 239: 487-491). In a first step, one hundred nongrams of genomic DNA was used in a reaction mixture containing each first round primer at a concentration of 2 μM/10 mM Tris pH 8.3/50 mM KCl/1.5 mM $MgCl_2$/0.1% gelatin/0.05 units per μl Taq DNA Polymerase (AmpliTaq, Perkin Elmer Cetus, Norwalk, CT). Reactions were assembled and incubated at 94°C for 1.5 minutes, followed by 30 cycles of 94°C/1 minute,

60°C/2 minutes, 72°C/3 minutes. Single stranded DNA was prepared in a second "asymmetric" PCR in which the products of the first reaction were diluted 1/1000. One of the primers was used at the standard concentration of 2 μM while the other was used at 0.08 μM. Under these conditions, both single stranded and double stranded molecules were synthesized during the reaction.

Solid phase immobilization of nucleic acids. GBA reactions wee performed in 96-well plates (Nunc Nuclon plates, Roskilde, Denmark). The GBA primer was covalently coupled to the plate by incubating 10pmoles of primer having a 5' amino group per well in 50 μl of 3 mM sodium phosphate buffer, pH 6, 20 mM 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) overnight at room temperature. After coupling, the plate was washed three times with 10mM Tris pH 7.5/150 mM NaCl/.05% polyoxyethylenesorbitan-20 (TWEEN-20) ("TNTw").

Biotinylated ddNTPs. Biotinylated ddNTPs were synthesized according to U.S. Patent No. 5,047,519.

GBA in Microwell Plates. Hybridization of single-stranded DNA primers covalently coupled to 96-well plates was accomplished by adding an equal volume of 3M NaCl/50 mM EDTA to the second round asymmetric PCR and incubating each well with 20 μl of this mixture at 55°C for 30 minutes. The plate was subsequently washed three times with TNTw. Twenty (20) μl of polymerase extension mix containing ddNTPs (3 μM each, one of which was biotinylated/5mM DTT/7.5 mM sodium isocitrate/ 5 mM MnCl2/0.04 units per μl of modified T7 DNA plymerase and incubated for 5 minutes at room temperature. Following the extension reaction, the plate was washed once with TNTw. Template strands were removed by incubating wells with another 50 μl 0.2N NaOH for 5 minutes at room temperature, then washing the wells with another 50 μl 0.2N NaOH. The plate was then washed three times with TNTw. Incorporation of biotinylated ddNTPs was measured by an enzyme-linked assay. Each well was incubated with 20 μl of strevavidin-conjugated horseradish peroxidase (1/1000 dilution in TNTw of product purchased from BRL, Gaitherburg, MD) with agitation for 30 minutes at room temperature. After washing

5 times with TNTw, 100 µl of o-phenylenediamine (OPD, 1mg/ml in 0.1 M Citric acid, pH 4.5 (BRL) containing 0.012% H_2O_2 was added to each well. The amount of bound enzyme was determined by photographing the plate after stopping the reaction or quantitatively using a Molecular Devices model "Vmax" 96-well spectrometer.--

After the Drawings, please insert the enclosed sequence listing.

IN THE CLAIMS

Please cancel claims 1-31 without prejudice. Please add the following new claims 32-45:

32. A method for identifying single nucleotide polymorphic sites in a genome of a species of interest, comprising:

(a) isolating a plurality of DNA fragments from the genome of a population of individual representatives of the species of interest, wherein each fragment corresponds to a location of the genome and the fragments are between about 0.1 kb and 10.0 kb;

(b) sequencing the DNA fragments to determine the nucleotide sequences of each fragment, and

(c) comparing the sequence of each fragment to corresponding fragments from other individual representatives of the species of interest to identify sites of sequence variation.

33. A method according to claim 32, wherein the plurality of DNA fragments is from a population of between 10 to 100 individual representatives of the same species.

34. A method according to claim 32, wherein the plurality of DNA fragments is from a population of between 100 to 1000 individual representatives of the same species.

35. A method according to claim 32, wherein the plurality of DNA fragments is from a population of between 100 and 10,000 individual representatives of the same species.

36. A method according to claim 32, wherein the fragments are between 0.5 kb and 3.0 kb.

37. A method according to claim 32, wherein the fragments are sequenced by dideoxy sequencing.

38. A method according to claim 32, wherein the fragments are isolated by amplification using oligonucleotide primers.

39. A method for determining allelic frequency at a single nucleotide polymorphic site, comprising:

(a) isolating a plurality of DNA fragments from a population of two or more individual representatives of a species of interest, wherein each fragment corresponds to a location of the genome and the fragments are between about 0.1 kb and 10.0 kb;

(b) sequencing the DNA fragments to determine the nucleotide sequences of each fragment;

(c) comparing the sequence of each fragment to corresponding DNA fragments from different individual representatives of the species of interest and identifying single nucleotide polymorphic sites having at least two alleles,

(d) determining the base identity of each allele present in the location of the genome, and

(e) calculating the allelic frequency for each allele by dividing the frequency at which each allele appears in the sample set by the total number of individuals.

40. A method according to claim 39, wherein the plurality of DNA fragments is from a population of between 10 to 100 individual representatives of the same species.

41. A method according to claim 39, wherein the plurality of DNA fragments is from a population of between 100 to 1000 individual representatives of the same species.

42. A method according to claim 39, wherein the plurality of DNA fragments is from a population of between 100 and 10,000 individual representatives of the same species.

43. A method according to claim 39, wherein the fragments are between 0.5 kb and 3.0 kb.

44. A method according to claim 39, wherein the fragments are sequenced by dideoxy sequencing.

45. A method according to claim 39, wherein the fragments are isolated by amplification using oligonucleotide primers.

REMARKS

The present application is a divisional application directed to the restricted claims of group VI from the office action dated May 5, 2000 of the parent application Serial No. 08/971,344. Claims 32-45 are now pending in the application. A Continued Prosecution Application (CPA) for the parent application Serial No. 08/971,344 was filed on April 17, 2001. Thus the present divisional application is filed herewith before the patenting, abandonment or termination of proceedings on application Serial No. 08/971,344.

If resolution of any remaining issue is required prior to examination of the application, it is respectfully requested that the Examiner contact Applicants' undersigned attorney at the telephone number provided below.

Respectfully submitted,

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